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(54) Title: METHOD FOR THE COMMERCIAL PRODUCTION OF TRANSGENIC PLANTS

(57) Abstract

A process for commercially propagating plants by tissue culture in such a way as both to conserve desired plant morphology and to transform the plant with respect to one or more desired genes. The method includes the steps of (a) creating an *Agrobacterium* vector containing the gene sequence desired to be transferred to the propagated plant, preferably together with a marker gene; (b) taking one or more petiole explants from a mother plant and inoculating them with the *Agrobacterium* vector; (c) conducting callus formation in the petiole sections in culture, in the dark; and (d) culturing the resulting callus in growth medium containing a benzylamino growth regulator such as benzylaminopurine or, most preferably, benzylaminopurine-riboside. Additional optional growth regulators including auxins and cytokinins (indole butyric acid, benzylamine, benzyladenine, benzylaminopurine, alpha naphthylacetic acid and others known in the art) may also be present. Preferably, the petiole tissue is taken from *Pelargonium x domesticum* and the *Agrobacterium* vector contains an antisense gene for ACC synthase or ACC oxidase to prevent ACC synthase or ACC oxidase expression and, in turn, the ethylene formation for which these enzymes are precursors.

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METHOD FOR THE COMMERCIAL PRODUCTION OF TRANSGENIC PLANTS

Statement Regarding Federally Funded Research

This invention was made with support from the Government under Experiment Station (Hatch Act) Project Nos. 2809 and 3179 awarded by the United States Department of Agriculture. The Government has certain rights in the invention.

Field of the Invention

This invention relates to the development of techniques for the commercial production of transgenic plants.

Background of the Invention

The genetic manipulation of plants is centuries old, and modern crop yields and disease- and pest-resistances often owe much to traditional plant genetic engineering. Classical plant breeding methods are time-consuming and subject to chance, however, so the recent advent of recombinant DNA techniques is promising. This promise is encouraging especially with respect to enabling plant geneticists to identify and to clone specific genes for desirable traits, and to introduce such genes into already useful varieties of plants.

Translating genetic engineering theory into practice, however, and then furthermore into a commercially practical reality, requires ingenuity. Gene transplantation in plants has already been accomplished at this writing--and examples are cited below--but heretofore no practical method for the commercial production of transgenic plants has been perfected.

Apart from the transgenic plant technology *per se*, it is known to propagate plants by replicating plant cells in culture, or "tissue culture." An early motivating force in the development of tissue culture was the desire to improve upon the relatively slow and low yields of vegetative propagation with the quick and exponential

proliferation of new plants from cell culture. Tissue culture methods are made possible by the plant physiological phenomenon of callus formation. When a plant is wounded, a patch of soft cells called a calli grows over the wound and, with time, phenolic compounds accumulate in the soft cells and harden, effectively sealing the wound. While hardened callus is the plant equivalent of scar tissue, callus is different from mammalian scar tissue with respect to its regenerative properties. If a piece of young, still-soft callus is removed and placed in a culture medium containing salts, sugars, vitamins, amino acids and the appropriate plant growth hormones, rather than harden, the cells will continue to divide and give rise to a disorganized mass of undifferentiated cells called a "callus culture." Plant or seedling "explants," or tissue samples, will likewise grow into similar cell cultures. The cultured cells can further be induced to redifferentiate into shoots, roots or whole plants by further culturing with the necessary hormones and growth media.

One of the most serious drawbacks with tissue culture propagation techniques has been the morphologic variation from generation to generation, a problem which is particularly notable in certain species and varieties. For example, as reported in Cassells, A.C., and Carney, B.F., "Adventitious regeneration in Pelargonium x domesticum Bailey," Acta Horticulturae, 212(II), 419-425 (1987), in stem and petiole tissue cultures of Grand Slam (as an example of P. domesticum, also known as Regal Pelargoniums or "Martha Washington" geraniums), up to 16% of the adventitious regenerants were variants, depending on the explant origin. The authors concluded that genome instability in Grand Slam and presumably other P. domesticum varieties may produce useful variation but mitigates against the use of adventitious regeneration in micropropagation.

The findings of Cassells et al. are consistent with the earlier work of Skirvin, R.M. and Janick, Jules, "Tissue Culture-Induced Variation in Scented Pelargonium ssp.," J. Amer. Soc. Hort. Sci., 101(3), 281-290 (1976). Skirvin et al. compared tissue culture propagated Pelargonium plants (from root cuttings, petiole cuttings or calliclones) with plants derived from vegetative propagation, i.e., stem cuttings. The plants derived from stem cuttings were all uniform and identical to the parental clone, whereas those from the root cuttings, petiole cuttings or calliclones were all morphologically distinct with the degree of variability depending upon the cultivar. The authors conclude that the variability associated with calliclones derived from tissue culture is a pool on which selection can be imposed, implying conversely that tissue culturing of this type is inappropriate for use in attempting reliable regeneration of Pelargonium x domesticum varieties.

Other varieties and species, besides Pelargonium x domesticum, are known and/or believed to suffer morphologic variation when propagated using tissue culture. It can be easily appreciated that any substantial morphologic variation in propagation is unacceptable for commercial propagation of a desired variety or species. Thus, tissue culture methods are not always acceptable for commercial use, even with the potentially much larger yields achievable as compared with prior art vegetative propagation techniques.

Apart from tissue culture considerations, gene transplantation in plants has achieved some success at this writing. Gene introduction is generally accomplished with a vector such as Agrobacterium. As this technology developed, it was noted that Crown Gall tumors of plants arose at the site of infection of some species of the bacterium Agrobacterium. The cells of Crown Galls acquire the properties of independent, unregulated growth. In culture, such transformed cells grow in the absence of the

plant hormones usually necessary for plant cell growth, and the cells retain the transformed phenotype even in the absence of the bacterium. The tumor-inducing agent in Agrobacterium is a plasmid that integrates some of its DNA into the chromosome of the host plant cells. Ti (tumor-inducing) plasmids exist in Agrobacterium cells as independently replicating genetic units.

Ti plasmids are maintained in Agrobacterium because part of the plasmid DNA, the T-DNA, carries the genes coding for the synthesis of amino acids called opines. The infected plant cell is induced to synthesize these amino acids, but the plant cannot use these amino acids. The Ti plasmid is believed to carry genes coding for enzymes that can degrade opines. Thus, Ti plasmids both make and degrade opines, within the plant cell, which the plant cell cannot metabolically use--presumably giving a selective advantage to the Agrobacterium at least with respect to utilization of the opine metabolites. A second set of genes in T-DNA codes for enzymes which lead to production of hormones which, in turn, cause the infected plant cell to divide in an unregulated way.

In summary terms, T-DNA enters a plant cell by what amounts to the equivalent of bacterial conjugation between the Agrobacterium and the plant cell. In other words, an Agrobacterium organism and a plant cell transfer their DNA in a process analogous to mating. Ultimately, T-DNA becomes incorporated into the genomic plant cell DNA in the plant cell nucleus.

All of the above background illustrates how Agrobacterium species can serve well as vectors for genetic transformation of plant cells. Early gene transfer using Ti plasmids, T-DNA and Agrobacterium was accomplished by the cointegration method, in which T-DNA was first cloned into a standard E. coli cloning vector, and the plant gene was subsequently cloned into a second cloning site carried by the vector. This intermediate vector was introduced into Agrobacterium organisms containing intact Ti plasmids.

Recombination occurred between the homologous regions of the intermediate vector and the wild-type Ti plasmid, and on infection of a plant with the <u>Agrobacterium</u> the recombinant plasmid is transferred to the plant cells.

Despite the early use of the cointegration method described above--and certainly it still works--the standard method for T-DNA transfer as of this writing is called the "binary system." The binary system was devised when investigators realized that the essential functions for transfer are supplied separately by the T-DNA itself and by the Ti plasmid, and that the components can be carried on separate vectors. The binary vector contains the borders of the T-DNA--needed for excision and integration--and the hormone-producing region of the original T-DNA can be removed and replaced with the foreign gene sequence intended for transfer to the plant cell. One side benefit of the use of binary vectors is that, by removing the hormone-producing regions of the T-DNA, uncontrolled growth of the recipient cells is prevented--or in other words the tumor-causing aspect of the T-DNA is nullified. The vir genes of the Ti plasmid can be supplied on a separate plasmid and etc.; the binary vector technique for gene transfer into plants is well established at this writing.

An example of the use of binary vectors to introduce functional genes into plants came about through experiments to use antisense RNA to control plant gene expression. Early work used binary vectors to introduce antisense polygalacturonase genes into tomato plants, to turn off the polygalacturonase expression which in turn digests pectin, in attempts to reduce bruising of tomato fruit during shipment. The results of these trials were disappointing. However, when binary vectors have been used to transfer antisense ethylene precursor genes into tomato plants, the results have been favorable. The antisense gene prevents expression of the ethylene precursor, no

ethylene production occurs during storage of the harvested tomatoes, and thus no ripening occurs until the time ripening is desired, when the fruit can be contacted with ethylene from another source.

Exemplary publications and patents which disclose transgenic plants and various techniques therefor are summarized below.

Pellegrineschi, A., et al., "Improvement of Ornamental Characters and Fragrance Production in Lemon-scented Geranium Through Genetic Transformation by *Agrobacterium rhizogenes*," *Bio/Technology*, Vol. 12 (January, 1994) discloses transformation of root cultures by inoculating stem and leaf fragments with *Agrobacterium rhizogenes*. An important plasmid in this species of *Agrobacterium* is the root-inducing plasmid which can be used to transfer to the plant genome the genes necessary for improved root growth in culture. The use of sterilized petioles as the source of explant material for plant transformation and culture is disclosed.

U.S. Patent No. 5,276,268 to Strauch et al., entitled "Phosphinothricin-Resistance Gene, and Its Use," is directed to the transfer of phosphinothricin-resistance gene into plants using *Agrobacterium* species. A modification of the binary vector method is discussed, and the phosphinothricin-resistance gene nucleic acid sequences are provided.

U.S. Patent No. 5,283,184 to Jorgenson et al. is entitled "Genetic Engineering of Novel Plant Phenotypes" and discusses transgenote formation and propagation in tissue culture, as well mentioning Pelargoniums and geraniums (and many other plants) by name. The tissue culture propagation of morphologically conserved transgenotes is not discussed.

U.S. Patent No. 5,286,635 to Hanson et al., entitled "Genetically, Transformed Pea Plants and Methods for Their Production," discloses the transfer of desired gene sequences into pea plants by incubating pea plant explants (preferably not callus) with Agrobacterium vectors containing the desired gene sequence. Mature seed material is used as the explant source. The issue of total morphologic conservation is not addressed.

Thus while certain inroads have been made in the area of tissue culture plant propagation as well as in plant gene transfer, a need remains for a method for the commercially viable production of transgenic plants in which the plants undergo only minimal, and thus commercially acceptable, morphologic variation as a result of tissue culture propagation.

Summary of the Invention

In order to meet this need, the present method is a process for commercially propagating plants by tissue culture in such a way as both to conserve desired plant morphology and to transform the plant with respect to one or more desired genes. The method includes the steps of (a) creating an Agrobacterium vector containing the gene sequence desired to be transferred to the propagated plant, preferably together with a marker gene; (b) taking one or more petiole explants from a mother plant and inoculating them with the Agrobacterium vector; (c) conducting callus formation in the petiole sections in culture, in the dark; and (d) culturing the resulting callus in growth medium having a benzylamino growth regulator such as

benzylaminopurine or, most preferably, benzylaminopurine-riboside. Additional optional growth regulators including auxins and cytokinins (indole butyric acid, benzylamine, benzyladenine, benzylaminopurine, alpha naphthylacetic acid and others known in the art) may also be present. Preferably, the petiole tissue is taken from <u>Pelargonium x domesticum</u> and the <u>Agrobacterium</u> vector contains an antisense gene for ACC synthase or ACC oxidase to prevent ACC synthase or ACC oxidase expression and, in turn, preventing ethylene formation. <u>Pelargoniums</u> propagated in culture using the present technique are resistant to wilting and petal shatter, and are morphologically conserved due to the use of petiole explants specifically and the particular culture media disclosed.

<u>Detailed Description of the Invention</u>

The present method is a process for propagating plants by tissue culture in such a way as both to conserve desired plant morphology and to transform the plant with respect to one or more desired genes. The method includes the steps of (a) creating an <u>Agrobacterium</u> vector containing the gene sequence desired to be transferred to the propagated plant, preferably together with a marker gene; (b) taking one or more petiole explants from a mother plant and inoculating them with the <u>Agrobacterium</u> vector; (c) conducting callus formation in the petiole sections in culture, in the dark; and (d) culturing the resulting callus in growth medium having a benzylamino growth regulator such as benzylaminopurine or, most preferably, benzylaminopurineriboside. Additional optional growth regulators including auxins and cytokinins (indole butyric acid, benzylamine, benzyladenine, benzylaminopurine, alpha naphthylacetic acid and others known in the art) may also be present. Preferably, the petiole tissue is taken from <u>Pelargonium x domesticum</u> and the <u>Agrobacterium</u> vector contains an antisense gene for ACC synthase or ACC oxidase to prevent ACC synthase or ACC oxidase expression and, in

turn, prevents ethylene formation. _Pelargoniums_ propagated in culture using the present technique are resistant to wilting and petal shatter, and are morphologically conserved due to the use of petiole explants specifically and the particular culture media disclosed.

Although in theory any anatomic explants can be mixed with _Agrobacterium_ containing the desired gene sequences to be transferred, followed by tissue culture propagation of transgenic transformed plants, in practice we have encountered unexpectedly good results using petioles as the explant tissue. We have found that morphologic conservation is virtually assured with the use of leaf petiole tissue, whereas morphologic variation--even between two generations--can result when explants of other tissue, i.e. leaf tissue, are used. Moreover, the petiole explants should be taken from stock plants (mother plants) of which commercial propagation is desired. Commercial viability is attributable to the large number of transgenically transformed plants which can be produced from a relatively few petioles taken from the mother plant--particularly because leaf petioles can be harvested from a mother plant with impunity, without endangering the mother plant.

The process of the present invention generally proceeds as follows. Leaves are harvested from stock plants for which commercial propagation is desired. The petiole section of each leaf is sterilized with a soap-and-water wash followed by surface sterilization using a solution containing soap and hypochlorite bleach, or a sequence of ethanol and bleach rinses. A good sterilization protocol rinses the petiole tissue in 70% aqueous ethanol for 1 minute, followed by a 15 minute rinse with 10% aqueous bleach, followed by two rinses with sterile water.

After sterilization, the leaf petioles are cut into approximately 1 cm pieces. The cut leaf petioles are inoculated with Agrobacterium cells which contain the gene sequence desired to be transferred to the plant cells, preferably together with a marker gene such as the kanamycin resistance gene known in the art. The inoculation can be as simple as the physical mixing of the cut leaf petioles with the Agrobacterium cells, with an approximate 30 minute incubation at ambient room or greenhouse temperatures.

Those skilled in the art know the significance of the use of a marker gene, but it is instructive to review that technology here. If a genetic sequence to be transplanted includes both the gene (or antisense gene) of interest adjacent a marker gene such as an antibiotic-resistance gene, the successfully genetically transformed cells can easily be separated from any cells in which the desired transformation did not occur. As a practical matter in plant propagation, a number of explants or other regenerative plant cells can be exposed to the gene/marker gene combination and then screened for successful transformants by, for example, inducing and growing the plantlets in culture medium containing the antibiotic for which the marker gene imparts resistance. If any plant grows in the antibiotic-containing medium, it will also have been transformed with respect to the desired gene adjacent the antibiotic-resistance gene. Explants or other cells which may not have underwent genetic transformation merely die in the culture medium--due to antibiotic susceptibility--and disappear.

Those skilled in the art also understand the significance of "antisense" molecular biology, but it should be borne in mind that primarily the present invention is intended to create transformants having antisense genes per se, and preferably not organisms containing vector-borne antisense mRNAs to prevent transcription of intact, or non-antisense, genes.

Transformation to create antisense genes is known in the art as exemplified by van der Krol, et al., "Antisense Chalcone Synthase Genes in Petunia Visualization of Variable Transgene Expression," Mol. Gen. Genet. (Molecular & General Genetics) Vol. 220, No. 2, pp. 204-212 (1990).

The inoculated petiole sections are then transferred to separate test tubes or vials containing culture medium. The culture medium contains vitamins, minerals, a food source and at least one growth regulator. The food source usually includes the Murashige Skoog salt known in the art, and preferably also includes additional food/energy sources, most preferably fresh coconut milk, as well as *Agrobacterium* virulence enhancers such as acetosyringone. An essential growth regulator is a benzylamino compound chemically equivalent to the most preferred benzylaminopurineriboside or the benzylaminopurines generally. The use of this class of growth regulators gives unexpectedly good results over the use of other growth regulators such as 2,4-dichlorophenoxyacetic acid, kinetin, gibberellic acid, abscisic acid or 6- -dimethylallylaminopurine (N^6 - [2-isopentenyl] adenine). Additional auxin and/or cytokinin growth regulators (indole acetic acid, indole butyric acid, benzylamine, benzyladenine, additional benzylaminopurine, alpha naphthylacetic acid and others) may also be present if they are in addition to, and not in substitution for, the benzyl/amino growth regulator selected.

The test tubes or vials are maintained for five days to two weeks in complete darkness, at a temperature of about 25° C. Over the five day to two week period, the section enlarges slightly and the ends form callus. Miniature shoots start forming intermittently on the callused ends of the petiole section.

After five days to two weeks, the enlarged petiole section bearing the miniature shoots is transferred from the test tube or vial to a Magenta vial or box known in the art. The enlarged petiole sections are housed five-

to-a-Magenta vial. The same growing medium as was originally charged to the test tube or vial is likewise charged to the Magenta vial, and in any event coconut milk should be present in the culture medium at this stage of the process. Also added to the medium is kanamycin (assuming the Agrobacterium contained the kanamycin resistance gene) and carbenicillin to kill any excess Agrobacterium. The Magenta vials are then maintained, under the same conditions as were the test tubes or vials, for an additional five to eight weeks in the dark and at about 25° C. The Magenta vials are then exposed to 5-10 weeks of 16 hours of light daily, in which the temperature is maintained at 72° F with 690 foot candles (6900 lux) of cool fluorescent light. During this time the petiole sections grow into enlarged clumps; the shoots elongate and turn into plantlets and many more shoots form. Once plantlets appear, they are transferred to fresh media containing kanamycin, carbenicillin and no growth hormones.

After the total growth period has elapsed, the clumps are removed and placed in sterile water. The individual plants are dissected out of the clump with a sterile scalpel. Each individual plant essentially has a series of leaves and nodes and is at least 1/2" high, but usually no roots are present. The individual plants are placed in RUBBER DIRT™ or other soil or soil-like growth media or growth media plugs, where rooting then takes place. Many varieties of Pelargonium x domesticum have been successfully tissue cultured through leaf petioles and multiplied, both with and without transgenic transformation via Agrobacterium. Morphologic variation has been minimal and within commercially acceptable limits for finished plant material. Other plants may be propagated by this tissue culture technique/transgenic technique also.

The creation of the Agrobacterium cell containing the desired vector can be accomplished by means known in the art. Structural and regulatory genes to be inserted may be obtained from depositories, such as the American

Type Culture Collection, Rockville, MD, 20852, as well as by isolation from other organisms, typically by the screening of genomic or cDNA libraries using conventional hybridization techniques. Typical hybridization techniques are dislosed in Sambrook, et al., Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989). Screening may be performed by (1) nucleic acid hybridization using homologous genes or heterologous genes from other organisms, (2) probes synthetically produced to hybridize to particular sequences coding for desired protein sequences, or (3) DNA sequencing and comparison to known sequences. Sequences for specific genes may be found in various computer databases, including GenBank, National Institutes of Health, or the database maintained by the United States Patent and Trademark Office.

The genes of interest may also be identified by antibody screening of expression libraries with antibodies made against homologous proteins to identify genes encoding for homologous functions. Transposon tagging can also be used to aid the isolation of a desired gene. Transposon tagging typically involves mutation of the target gene. A mutant gene is isolated in which a transposon has inserted into the target gene and altered the resulting phenotype. Using a probe for the transposon, the mutated gene can be isolated. Then, using the DNA adjacent to the transposon in the isolated, mutated gene as a probe, the normal wild-type allele of the target gene can be isolated. Such

techniques are taught, for example, in McLaughlin and Walbot, Genetics, Vol. 117 pp. 771-776 (1987), as well as numerous other references.

In addition to the functional gene and the selectable marker gene, the DNA sequences may also contain a reporter gene which facilitates screening of the transformed shoots and plant material for the presence and expression of endogenous DNA sequences. Exemplary reporter genes include ß-glucuronidase and luciferase.

As described above, the exogenous DNA sequences are introduced into the area of the explants by incubation with Agrobacterium cells which carry the sequences to be transferred within a transfer DNA (T-DNA) region found on a suitable plasmid, typically the Ti plasmid. Ti plasmids contain two regions essential for the transformation of plant cells. One of these, the T-DNA region, is transferred to the plant nuclei and induces tumor formation. The other, referred to as the virulence (vir) region, is essential for the transfer of the T-DNA but is not itself transferred. By inserting the DNA sequence to be transferred into the T-DNA region, introduction of the DNA sequences to the plant genome can be effected. Usually, the Ti plasmid will be modified to delete or to inactivate the tumor-causing genes so that they are suitable for use as a vector for the transfer of the gene constructs of the present invention. Other plasmids may be utilized in conjunction with Agrobacterium for transferring the DNA sequences of the present invention to the plant cells.

The construction of recombinant Ti plasmids may be accomplished using conventional recombinant DNA techniques, such as those described by Sambrook et al. (1989). Frequently, the plasmids will include additional selective marker genes which permit manipulation and construction of the plasmids in suitable hosts, typically bacterial hosts other than Agrobacterium, such as E. coli. In addition to the above-described kanamycin resistance

marker gene, other exemplary genes are the tetracycline resistance gene and the ampicillin resistance gene, among others.

The genes within the DNA sequences will typically be linked to appropriate transcriptional and translational control sequences which are suitable for the <u>Pelargonium</u> plant host. For example, the gene will typically be situated at a distance from a promoter corresponding to the distance at which the promoter is normally effective in order to ensure transcriptional activity. Usually, a polyadenylation site and transcription termination site will be provided at the 3'-end of the gene coding sequence. Frequently, the necessary control functions can be obtained together with the structural gene when it is isolated from a target plant or other host. Such intact genes will usually include coding sequences, intron(s), a promoter, enhancers, and all other regulatory elements either upstream (5') or downstream (3') of the coding sequences.

The binary vector system generally discussed above may be used to introduce the DNA sequence according to the present invention. A first plasmid vector strain would carry the T-DNA sequence while a second plasmid vector carries a virulence (vir) region. By incubating <u>Agrobacterium</u> cells carrying both plasmids with the explant, infection of the plant material is thus achieved.

Any one of a number of T-DNA plasmids can be used with such a binary vector system, the only requirement being that one be able to select independently for the two plasmids. The T-DNA plasmid in a preferred embodiment comprises a heterologous promoter which promotes the transcription of one or more genes within the exogenous DNA fragment(s). An example is the Cauliflower Mosaic Virus 35S promoter (Odell et al., <u>Nature</u>, Vol. 313, pp. 810-812 (1985) among others.

Suitable Agrobacterium species include Agrobacterium tumefaciens and Agrobacterium rhizogenes. While the wild-type Agrobacterium rhizogenes may be used, the Agrobacterium tumefaciens should be disarmed by deactivating its tumor activating capacity prior to use. Preferred Agrobacterium tumefaciens strains include LBA4404, as described by Hoekema et al., Nature, Vol. 303, pp. 179-180 (1983) and EHA101 as described by Hood et al., J. Bacteriol., Vol. 168, pp. 1291-1301 (1986). A preferred Agrobacterium rhizogenes strain is 15834, as described by Birot et al., Plant Physiol. Biochem., Vol. 25, pp. 323-325 (1987).

As the Agrobacterium strains carrying the desired exogenous DNA sequences are being prepared, the following antisense sequences are preferred for use in the present process, and are particularly preferred in transforming regal Pelargonium petiole explants according to the present method. These sequences are the known sequences for ACC Synthase (1-aminocyclopropane-1-carboxylate synthase) and ACC Oxidase (1-aminocyclopropane-1-carboxylate oxidase), reversed to create antisense sequences. Tables 1 and 2 show two separate ACC Synthase genes labeled SEQ ID NO:1: and SEQ ID NO:2: respectively. Table 3 shows ACC oxidase gene sequence labeled SEQ ID NO:3:.

After the Agrobacterium strains carrying the desired exogenous DNA sequences have been prepared, they will usually be cultured for a period of time prior to incubation with the explant material. Initially, the Agrobacterium may be cultured on a solid media including nutrients, an energy source and a gelling agent. Suitable nutrients include salts, tryptone and yeast extracts, while most sugars are suitable as the energy source and the gelling agent can be TC agar or bactoagar or other similar products. The Agrobacterium cells are typically cultured for about two to five days, preferably in the dark at about 23-28° C, and are collected by scraping before browning (while still a white color). The cells are scraped from

the medium and suspended in a liquid medium such as L-broth, pH 6.9-7.1, preferably 7.0. The bacteria are cultured in liquid medium for 8-36 hours, preferably 12-20, on a shaker (50-220 rpm, preferably 100-120 rpm) at 23-28° C. At the end of this period the bacteria are diluted to an optical density of 0.3 and cultured for 2-6, preferably 4, hours (on a shaker, 23-28° C). The Agrobacterium cells thus incubated are ready for inoculation onto explant material such as Pelargonium petiole sections.

When Agrobacterium cells are inoculated onto Pelargonium x domesticum petiole explants, and after the coincubation discussed above, the tissue culture method will proceed generally in accordance with the disclosure of U.S. Application Serial No. 08/149,702 filed November 9, 1993, which is a Continuation of U.S. Application Serial No. 07/690,073 filed April 23, 1991, by Wendy Oglevee-O'Donovan and Eleanor Stoots, both of which applications are hereby incorporated herein by reference. By means known in the art, carbenicillin can be added to the coincubated petiole/Agrobacterium cells to kill excess Agrobacterium cells, after transfection has taken place. The key is to select an antibiotic which will kill the Agrobacterium without harming the explant material. An additional amount of the same antibiotic may be provided in the ensuing tissue culture method, to assure final removal of any viable Agrobacterium cells.

After green transformed shoots are approximately 1/2" tall, they can then be transplanted to soil within a greenhouse or elsewhere in a conventional manner for tissue culture plantlets. Transformation of the resulting plantlets can be confirmed by assaying activity for the selection marker, or by assaying the plant material for any of the phenotypes which have been introduced by the exogenous DNA. Suitable assay techniques include polymerase chain reaction (PCR), restriction enzyme digestion, Southern blot hybridization and Northern blot hybridization.

The present invention represents a breakthrough in the commercial production and genetically transformed plants. Because the method uses petiole tissue from a grower's mother plant (a stock plant), the starting petiole explants have a commercially desirable morphology to begin with--by definition. However, if the mother plant could be improved by genetic transformation of some type, for example to deactivate a gene which expresses an enzyme in the ethylene synthesis pathway, the progeny of the mother plant may thus be improved in this one way over their parent stock. The petiole tissue from the stock plant, plus the genetic transformation from the Agrobacterium, yield both an improved genetic makeup of the commercially produced plants--although with preserved desired morphology from the mother plant--and at the same time the high yields possible only with the generation of many plantlets in a single generation's growth in tissue culture. In summary, with the present method a single genetically transformed mother plant can yield literally thousands of offspring plants. No one in the prior art has attempted to combine these two previously disparate technologies to achieve a unique method in which the result is no less than a commercially viable technique for making genetically recombinant plants in commercially feasible numbers (See Example 4).

Example 1

Agrobacterium tumefaciens strain LBA4404 containing the binary vector pBI 121 which contains the Cauliflower Mosaic Virus 35S promoter with the nptII gene which confers kanamycin resistance and the nos terminator. The *Agrobacterium* cells were maintained on LB plates containing kanamycin and streptomycin. Overnight suspensions were initiated by the addition of a single

colony to 5 ml of LB broth and grown at 28° C on a shaker in the dark. This example illustrates the preparation of an inoculum from a commercially available binary vector containing Agrobacterium tumefaciens strain.

Example 2

An aliquot of the inoculum prepared in Example 1 was added to Pelargonium x domesticum explants as follows. Petioles were removed from Pelargonium x domesticum "Honey" plants and were surface sterilized in a solution containing 70% aqueous ethanol for 1 minute followed by washing in 5% aqueous sodium hypochlorite and 0.1% Tween 20 for 15 minutes. The petiole sections were then rinsed four times using sterile distilled water. Both ends (approximately 3 mm) of the petiole piece were removed and discarded. The remaining petiole was cut into 10 mm segments.

The petiole sections were moved to a flask with enough liquid cocultivation media amply to cover. The cocultivation medium contained vitamins, minerals, a food source, two growth regulators (four parts per million of benzylaminopurineriboside and ten parts per million of indole butyric acid) and sufficient acetosyringone to yield 200 μM acetosyringone. To this flask was charged the aliquot of Agrobacterium inoculum, and the flask was then swirled for approximately 15 minutes. Additional fresh cocultivation medium plus Agrobacterium inoculum, solidified onto culture plates, was used to receive the explants after pipetting off the liquid cocultivation medium. The plates were incubated in a growth chamber in the dark at 23° C for three days.

Example 3

After conducting Example 2, all explants were washed in liquid media supplemented with 500 μM cefotaxime for two 1 hour washes. The explants were then transferred to plates containing regeneration media (the cocultivation media minus the acetosyringone and the Agrobacterium), 200

mg/l kanamycin, and 500 mg/l carbenicillin solidified with 0.2% Gelrite. The petri dishes were wrapped in parafilm and grown in a growth chamber in the dark at 23° C. After 3-5 weeks, untransformed tissue became dark brown and died. Small "buds" appeared on transformed tissue after 4-6 weeks. After approximately 6-7 weeks tissue containing shoots was transferred to magenta vessels containing regeneration medium, 200 mg/l kanamycin, and 500 mg/l carbenicillin solidified with 0.2% Gelrite. The Magenta vessels were then transferred to a growth room and grown under periods of light and dark wherein 16 hours of dark was followed by 8 hours of light, then 16 hours of dark and so on. When the shoots were 2-3 cm tall, they were transferred to magenta vessels containing rooting media. Rooted shoots were then acclimated to a greenhouse environment. Six transformed plants were produced on selection medium. NPT assays, GUS assays, and Southern blot analysis were used for confirmation of transformed <u>Pelargoniums</u>.

<u>Example 4</u>

Using <u>Agrobacterium</u> containing the appropriate vectors, petiole explants from <u>Pelargonium x domesticum</u> are transformed for insertion of one of the following antisense genes: antisense ACC Synthase or antisense ACC Oxidase. Except for this alternate genetic transformation, the remaining steps proceed according to Examples 1-3.

Example 5

Forty petioles are taken from a transformed Pelargonium x domesticum stock plant according to Example 4. Each petiole is divided into 4 segments to make 160 explants. The explants are grown in culture according to Examples 1-3. With an average of 30 plantlets per cultured petiole segment, the total production from the transformed stock plant is thus 4800 offspring plants (160 x 30) without even beginning to exhaust the available petiole tissue from the stock plant.

TABLE 1 SEQ ID NO:1:

GAC-1

```
tatcactactctcgcttctgagtgcctaattattttgtccaagctctcagtacgtacgtgttgtacgtgtttacata
gATGGAGAACAAGAGCAAACAGCTTCTGTCAAAGATTGCAACCAACGACGGACACGGCGAGAACTCCCCATATTTCGA   156
  M  E  N  K  S  K  Q  L  L  S  K  I  A  T  N  D  G  H  G  E  N  S  P  Y  F  D

TGGTTGGAAGGCTTATGACCGTGATCCGTTCCATCCGTCTCAGAATCCTAACGGTGTTATCCAGATGGGTTTAGCTGA
  G  W  K  A  Y  D  R  D  P  F  H  P  S  Q  N  P  N  G  V  I  Q  M  G  L  A  E

OLE-2                                                                 OLE-5
AAATCAGCTTTCATCTGACTTGATTGAAGATTGGGTGAGGTCCAACCCAGAAGCCTCAATCTGCACTCTTGAGGGAGT   312
  N  Q  L  S  S  D  L  I  E  D  W  V  R  S  N  P  E  A  S  I  C  T  L  E  G  V

TGGTAAGTTCAAGGACGTAGCTAACTTTCAGGACTACCATGGCCTGCTGGAGTTCAGGCACGCCGTGGCTAAATTTAT
  G  K  F  K  D  V  A  N  F  Q  D  Y  H  G  L  L  E  F  R  H  A  V  A  K  F  M
                                   OLE-3
GAGCAGAGGAAGGGGCGGGAAGGTCACATTTGATCCCGACCGTGTCGTCATGAGCGGCGGAGCCACCGGAGCCAACGA   468
  S  R  G  R  G  G  K  V  T  F  D  P  D  R  V  V  M  S  G  G  A  T  G  A  N  E

GCTCATCGTCTTCTGTTTGGCCAATCCCGGCGACGCTTTCCTTCTCCCATCTCCTTATTATCCAGCAAACGACCGTGA
  L  I  V  F  C  L  A  N  P  G  D  A  F  L  I  P  S  P  Y  Y  P  A  N  D  R  D

CTTGCAGTGGCGAACCGGAGCTCAGATCATTCCGGTGCACTGCAACAGCTCCAACGGTTTCAAGATAACCAGAGAGGC   624
  L  Q  W  R  T  G  A  Q  I  I  P  V  H  C  N  S  S  N  G  F  K  I  T  R  E  A

ACTAGAAAGATCATACGCACAAGCACAAGAAAGCAACATAAACGTAAAAGGCGTGCTCTTAACCAACCCATCGAACCC

  L  E  R  S  Y  A  Q  A  Q  E  S  N  I  N  V  K  G  V  L  L  T  N  P  S  N  P

TCTAGACACAATTCTGGACCGCGACACTCTCAAGAGCATCGTCAGCTTCGTCACCGACAACAACATCCACCTAGTCAT   780
  L  G  T  I  L  D  R  D  T  L  K  S  I  V  S  F  V  T  D  N  N  I  H  L  V  I

CGACGAAATCTACGCCGCCACCGTTTTCGTTGCCCCGGAGTTCGTAAGCGTCTCCGAAATCCTCCAAGAAATGGACGA
  D  E  I  Y  A  A  T  V  F  V  A  P  E  P  V  S  V  S  E  I  L  Q  E  M  D  D

CACCACGTGCAACCCCGACCTCATCCACATCGTGTACAGCCTGTCCAAGGACTTGGGCATGCCCGGGTTCCGCGTCGG   936
  T  T  C  N  P  D  L  I  H  I  V  Y  S  L  S  K  D  L  G  M  P  G  F  R  V  G

GATCGTGTACTCATTCAACGACGACGTCGTATCCTGCGCACGGAAGATGTCGAGCTTCGGGTTGGTGTCGACCCAGAC
  I  V  Y  S  F  N  D  D  V  V  S  C  A  R  K  M  S  S  F  G  L  V  S  T  Q  T

GCAGCACCTTCTCGCAGCGATGCTATCCGACGACGTTTTCGTGGAGCGGTTCCTCGCGGAGCGGAGGCGGTTGGGGAG  1092
  Q  H  L  L  A  A  M  L  S  D  D  V  F  V  E  R  F  L  A  E  R  R  R  L  G  R

GAGGCACGGCGTGTTCACGAAAGGGCTCGAGGAGTTGGGGATTGGGTGTTTAAAGAGCAACGCGGGGCTCTACTTCTG
  R  H  G  V  F  T  K  G  L  E  E  L  G  I  G  C  L  K  S  N  A  G  L  Y  F  W
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```
GATGGATTTGCGGAAGCTTC[illegible]AGAAGAGACGTTTGAGGCGGAGATGGTGCTGTGGAAG[illegible]TTATTAATGAGGT   1248
 M  D  L  R  K  L [illegible] E  E  T  F  E  A  E  M  V  L  W  K [illegible] I  N  E  V

GAAGCTAAACGTGTCTCCGGGGTCGTCGTTTCATTGCGTGGAGCCGGGTTGGTTTAGGGTTTGCTTTGCCAACATGGA
 K  L  N  V  S  P  G  S  S  F  H  C  V  E  P  G  W  F  R  V  C  F  A  N  M  D
                                                                   OLE-4
                                                      OLE-6
CGACGAGACGGTCCACGTGGCGCTGAAGAGGATCAGGGCGTTTGTGAGGAAGAAGGAGGTGGGTCCGGTGAAGAGGAA   1404
 D  E  T  V  H  V  A  L  K  R  I  R  A  F  V  R  K  K  E  V  G  P  V  K  R  K

GAGGTTCATGGACAACCTTAACCTCAGGCTGAGCTTCTCGTCGCTAAGGTACGATGAGAGTGTGATGTTGTCGCCGCA
 R  F  M  D  N  L  N  L  R  L  S  F  S  S  L  R  Y  D  E  S  V  M  L  S  P  H

CATAATGGTGTCCACGCACTCGCCGCTTGTTCGTGCGAGAACAtaatgagcatgcacgtttttatttgctactgttag   1560
 I  M  V  S  T  H  S  P  L  V  R  A  R  T

taattaactaattaattgttatttgattgtgtgctgaatgttggattctttctttgtagaagagaagctataggagat
gtttttaaccaattaccgtagatatatatgcagtggaattaagaaaaatagaggttaaatattaattccatgcatata   1716
tatgtaggaaggaattggtacatatttagggtttgctgatgttttctttcatcatgaattggtacatatttatgatg
ttcaaggctccaagtgatggatacatggaggattcatttggatgcatgccttgcaagagtcagcaatctttgttaatt   1872
agtgtatggtttgtgataataaagatgcaaaattctgtgttgttttaaaaaaaaaaaaaaa                     1934
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TABLE 2 SEQ ID NO:2:

GAC-2

TATTTTGATGGGTGGAAGGCTTACGACAACAATCCTTTCCATCTCACCCAAAACCCTCAAGGTGTCATCCAGATGGGC
Y F D G W K A Y D N N P F H L T Q N P Q G V I Q M G
OLE-2
CTCGCAGAAAATCAGCTTTCTTTCGAGTTGATTGAGCAATGGGTCCTTAACAACCCACAAGCCTCCATTTGCACAGCA 156
L A E N Q L S F E L I E Q W V L N N P Q A S I C T A
OLE-5
CAAGGTCTGCAAGAATTCAAGGACACTGCAATCTTTCAAGATTACCATGGCTTGCCAGAGTTCAGATATGCTGTTGCA
Q G L Q E F K D T A I F Q D Y H G L P E F R Y A V A

AATTTCATGGGAAAGGTGAGAGGAAACAGAGTAACATTTAACCCAGATCGCATAGTTATGAGTGGAGGAGCAACTGGA 312
N F M G K V R G N R V T F N P D R I V M S G G A T G

GCTCATGAAATGATTGCCTTCTGTTTGGCTGATCCTGGCGATGCTTTTCTTGTCCCAACTCCTTATTATCCTGGATTT
A H E M I A F C L A D P G D A F L V P T P Y Y P G F

GATAGAGACCTGAGGTGGAGAACTGGTGTGCAGCTAATTCCTGTAGTCTGTGAAAGTGAAAACAATTTCAGGATCACC 468
D R D L R W R T G V Q L I P V V C E S E N N F R I T

CGAAGTGCCTTAGAAGAAGCCTATGAGAGAGCTCAAGAGGACAACATTAGAGTCAAGGGATTGCTCATAACAAACCCA
R S A L E E A Y E R A Q E D N I R V K G L L I T N P

TCAAACCCACTAGGAACTATCCTGGACAGAGAGACACTGGTCAGTCTAGTGAGCTTCATCAATGAAAAGAACATTCAC 624
S N P L G T I L D R E T L V S L V S F I N E K N I H

TTGGTCTGTGATGAAATCTACGCCGCCACAGTCTTCTCTCAGCCCGCTTTCGTTAGCATTGCTGAGGTTATCGAGCAA
L V C D E I Y A A T V F S Q P A F V S I A E V I E Q

GAGAACGTTTCGTGCAACCGCGACCTCATCCACATTGTCTACAGCCTGTCCAAGGACATGGGCTTCCCTGGCTTCAGG 780
E N V S C N R D L I H I V Y S L S K D M G F P G F R

GTGGGGATTGTCTACTCCTACAATGACGCAGTTGTGAATTGTGCGCGAAAGATGTCAAGTTTCGGCCTTGTATCCACA
V G I V Y S Y N D A V V N C A R K M S S F G L V S T

CAAACTCAGCACCTAATCGCATCAATGCTCTCGGACGATGAATTCGTGGACACATTCATCGTGGAGAGCGCGAAGAGG 936
Q T Q H L I A S M L S D D E F V D T F I V E S A K R

CTAGCGAGAAGGTACGCAACCTTCACAAGAGGGCTTGCACAAGTCCACATTGGGAGCCTAAAGAGCAATGGGGGGTTA
L A R R Y A T F T R G L A Q V H I G S L K S N G G L

TTCATATGGATGGACTTGAGGAGGCTTCTCAAGGAGAAGACTTTCGAGGCGGAGATGGCTCTGTGGAGAGTGATAATC 1092
F I W M D L R R L L K E K T F E A E M A L W R V I I

AATGAGGTGAAGCTAAATGTGTCGCCAGGGGCGTCGTTCCATTGCTCGGAGCCAGGGTGGTTCAGAGTCTGTTTCGCT 1170
N E V K L N V S P G A S F H C S E P G W F R V C F A
OLE-6

TABLE 3 SEQ ID NO:3:

GEFE-1

```
cttgagtcttgagtgtgtgttagcaagaaacaaacattagtgtgaaaacacaagagaaggagaaaaaaataccttgct
tttattggagATGGAGAGCTTTCCAGTGATCAACATGGAGAAGTTGAATGGTGAGGAGAGAGCAGCAACCATGGAGAA   156
           M  E  S  F  P  V  I  N  M  E  K  L  N  G  E  E  R  A  A  T  M  E  K

GATCAAGGATGCTTGTGAAAACTGGGGTTTTTTTGAGCTGTTGAACCATGGGATACCCTATGAGCTGCTTGACACAGT
 I  K  D  A  C  E  N  W  G  F  F  E  L  L  N  H  G  I  P  Y  E  L  L  D  T  V   49

GGAGAAGATGACAAAGGAGCATTACAGGAAGTGTATGGAGCAGAGGTTTAAGGAAATGGTGGCAAGCAAGGGACTTGA   312
 E  K  M  T  K  E  H  Y  R  K  C  M  E  Q  R  F  K  E  M  V  A  S  K  G  L  E

AGGAGTGGAGGTAGAGGTTGAGGACTTGGATTGGGAGAGCACTTTTTTTCTTGAAGCATCTCCCAGAATCAAACATCTC
 G  V  E  V  E  V  E  D  L  D  W  E  S  T  F  F  L  K  H  L  P  E  S  N  I  S   101

TCAAGTCCCTGATCTTCAAGACGAGTACAGGAAGGTGATGAAGGAATTTGCAGCAAAACTAGAGAAACTAGCCGAGGA   468
 Q  V  P  D  L  Q  D  E  Y  R  K  V  M  K  E  F  A  A  K  L  E  K  L  A  E  E

GCTACTAGACCTGTTGAGCGAGAATCTTGGGCTAGAGAAAGGTTACCTGAAAAAAGCTTTCTATGGCTCAAAGGGTCC
 L  L  D  L  L  S  E  N  L  G  L  E  K  G  Y  L  K  K  A  F  Y  G  S  K  G  P   153

AACCTTTGGCACCAAGGTCAGCAACTACCCTCCCTGCCCCAAGCCAGACTTAATCAAGGGACTCAGGGCACATACAGA   624
 T  F  G  T  K  V  S  N  Y  P  P  C  P  K  P  D  L  I  K  G  L  R  A  H  T  D

TGCCGGAGGCCTCATATTGCTCTTCCAAGACGACAAGGTCAGTGGTCTCCAGCTCCTGAAAGACGGGAAGTGGGTCGA
 A  G  G  L  I  L  L  P  Q  D  D  K  V  S  G  L  Q  L  L  K  D  G  K  W  V  D   205

TGTTCCTCCTATGCACCACTCCATCGTCATCAACCTCGGTGACCAACTTGAGGTGATTACCAATGGGAAATACAAGAG   780
 V  P  P  M  H  H  S  I  V  I  N  L  G  D  Q  L  E  V  I  T  N  G  K  Y  K  S

CATAGAGCACCGTGTGATAGCCCAATCAGACGGTACTAGAATGTCCATTGCTTCCTTCTACAACCCGGGAAGTGATGC
 I  E  H  R  V  I  A  Q  S  D  G  T  R  M  S  I  A  S  F  Y  N  P  G  S     D  A   257

GGTCATCTATCCAGCACCAGCTCTGTTGGAGAAAGAAACAGAAGAGAAGCAAGTGTACCCGAAATTCGTGTTCGAAGA   936
 V  I  Y  P  A  P  A  L  L  E  K  E  T  E  E  K  Q  V  Y  P  K  F  V  F  E  D

CTACATGAAGCTCTACTCTGGCCTCAAGTTCCAAGCCAAAGAGCCCAGATTTGAAGCCATGAAAGCTGTGGAGGCTAA
 Y  M  K  L  Y  S  G  L  K  F  Q  A  K  E  P  R  F  E  A  M  K  A  V  E  A  N   309

TGTTACTTTGGATCCAATTCGAACTGCCtagaaagatattatacaacaaccttagcagatcagaaagaagaagaacaa  1092
 V  T  L  D  P  I  R  T  A                                                       313

agggtagactgtgttgtctgttcttaaggtggttgtgttgtgtccaggctgctaaaagctttgtgatttgtttttaaa
ttttatgacgcacggcttactataatgggttctttatcagtttgtttatagtcatgggtgctaattatttggtattat  1248
aatatataagagtattagtcaaaaaaaaaaaaaaaaaaaaaaa                                      1291
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Although the invention has been described with particularity above, especially in the Examples, this disclosure is illustrative only and the invention is thus to be limited only insofar as is set forth in the accompanying claims.

What Is Claimed Is:

1. A method for commercially producing transgenic plants, comprising:

harvesting at least one petiole from a mother plant;

inoculating said petiole with a vector contained within an *Agrobacterium* bacteria and genetically transforming at least one cell in said petiole;

culturing the transformed petiole tissue thus produced in a culture medium containing at least one benzylaminoglycoside growth regulator and with exposure to periods of light and dark; and

removing small plants generated by the transformed petiole tissue to conditions which permit its further rooting and growth.

2. The method according to claim 1 wherein said culture medium contains benzylaminopurineriboside.

3. The method according to claim 1 wherein the *Agrobacterium* vector contains an antisense-ACC Synthase gene.

4. The method according to claim 1 wherein the *Agrobacterium* vector contains an antisense-ACC Oxidase gene.

5. The method according to claims 3-4 wherein said *Agrobacterium* vector further contains a marker gene.

6. A method for commercially producing transgenic *Pelargonium x domesticum* plants, comprising:

harvesting at least one petiole from a *Pelargonium x domesticum* mother plant;

inoculating said petiole with a vector contained within an *Agrobacterium* bacteria and genetically transforming at least one cell in said petiole;

culturing the transformed petiole tissue thus produced in a culture medium containing at least one benzylaminoglycoside growth regulator and with exposure to periods of light and dark; and

removing small plants generated by the transformed petiole tissue to conditions which permit its further rooting and growth.

7. The method according to claim 6 wherein said culture medium contains a growth regulator selected from the group consisting of benzylaminopurineriboside and benzylaminopurine.

8. The method according to claim 6 wherein the *Agrobacterium* vector contains an antisense ACC Synthase gene.

9. The method according to claim 6 wherein the *Agrobacterium* vector contains an antisense ACC Oxidase gene.

10. The method according to claims 8-9 wherein said *Agrobacterium* vector further contains a marker gene.

11. The method according to claim 10 wherein the inoculating step is conducted by cocultivating said petiole and said *Agrobacterium* in a medium containing benzylaminopurineriboside growth regulator.

12. The method according to claim 10 wherein said marker gene is the kanamycin resistance gene.

13. The method according to claim 12 wherein said *Agrobacterium* vector further contains a nucleic acid sequence which is antisense to the sense sequence of SEQ ID NO:1.

14. The method according to claim 12 wherein said *Agrobacterium* vector further contains a nucleic acid sequence which is antisense to the sense sequence of SEQ ID NO:2.

15. The method according to claim 12 wherein said *Agrobacterium* vector further contains a nucleic acid sequence which is antisense to the sense sequence of SEQ ID NO:3.

16. The product prepared in accordance with the method of claim 1.

17. The product prepared in accordance with the method of claim 6.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17954

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205, DIG 22; 435/69.1, 172.3, 240.4, 320.1; 536/23.6, 24.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CABA, CAPLUS, GENBANK, SCISEARCH
search terms: acc synthase, acc oxidase, ethylene, pelargonium, benzylaminopurine, petiole, agrobacterium

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERNST et al. Isolation and identification of a new, naturally occurring cytokinin (6-benzylaminopurineriboside) from an anise cell culture (Pimpinella anisum L.). Planta. 1983, Vol. 159, pages 222-225, see entire document.	1-17
Y	DUNBAR et al. Plant regeneration from callus-derived protoplasts of Pelargonium X domesticum. Plant Cell Reports. 1991, Vol. 10, pages 417-420, especially page 417.	1-17
Y	PELLEGRINESCHI et al. Improvement of Ornamental Characters and Fragrance Production in Lemon-scented Geranium Through Genetic Transformation by Agrobacterium rhizogenes. Bio/Technology. January 1994, Vol. 12, pages 64-68, see entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 JANUARY 1997	06 FEB 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	THOMAS HAAS
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17954

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROBICHON et al. Genetic transformation of Pelargonium X hortorum. Plant Cell Reports. 1995, Vol. 15, pages 63-67, especially pages 63-64.	1-17
Y	SAVIN et al. Antisense ACC Oxidase RNA Delays Carnation Petal Senescence. HortScience. August 1995, Vol. 30, No. 5, pages 970-972, see entire document.	1-17

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17954

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 5/04, 5/14, 9/00, 15/00, 15/05, 15/09, 15/29, 15/64, 15/82; A01H 1/00, 1/04, 4/00,

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

800/205, DIG 22; 435/69.1, 172.3, 240.4, 320.1; 536/23.6, 24.1, 24.5

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